

Repression of the vertebrate organizer by Wnt8 is mediated by Vent and Vox

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Summary

Dorsoventral (DV) patterning of vertebrate embryos requires the concerted action of the Bone Morphogenetic Protein (BMP) and Wnt signaling pathways. In contrast to our understanding of the role of BMP in establishing ventral fates, our understanding of the role of Wnts in ventralizing embryos is less complete. Wnt8 is required for ventral patterning in both *Xenopus* and zebrafish; however, its mechanism of action remains unclear. We have used the zebrafish to address the requirement for Wnt8 in restricting the size of the dorsal organizer. Epistasis experiments suggest that Wnt8 achieves this restriction by

regulating the early expression of the transcriptional repressors Vent and Vox. Our data show that *vent* and *vox* are direct transcriptional targets of Wnt8/ β -catenin. Additionally, we show that Wnt8 and Bmp2b co-regulate *vent* and *vox* in a dynamic fashion. Thus, whereas both Wnt8 and zygotic BMP are ventralizing agents that regulate common target genes, their temporally different modes of action are necessary to pattern the embryo harmoniously along its DV axis.

Key words: Wnt8, BMP, Dorsoventral, Vent, Vox

Introduction

Formation of the vertebrate embryonic axes requires Wnt signaling at two points: after fertilization, to establish a dorsal signaling center, and during gastrulation, to pattern and specify ventral fates (for reviews, see De Robertis et al., 2000; Schier, 2001). Although canonical Wnt/ β -catenin signaling is involved in both processes, it is triggered differently in each case. Specification of the dorsal signaling center appears to be a ligand-independent mechanism involving the accumulation of β -catenin, the nuclear effector of Wnt signaling, in dorsal nuclei (Larabell et al., 1997; Kelly et al., 2000; Schier, 2001). Accumulation of nuclear β -catenin leads to the formation of the Nieuwkoop center, which induces the dorsal mesodermal structure known as Spemann's Organizer (known as the 'shield' in zebrafish or the 'node' in the mouse) (for a review, see Moon and Kimelman, 1998). After the establishment of the dorsoventral (DV) axis, Wnt/ β -catenin activity stimulated by the ligand Wnt8 is required to antagonize the organizer; thus, zebrafish *wnt8* mutants, or *Xenopus* embryos expressing a dominant-negative Xwnt8, display enlarged organizers and concomitant loss of posterior and ventral tissues (Hoppler et al., 1996; Lekven et al., 2001). Because proteins secreted by the organizer are known to be required for head formation and embryonic patterning (for a review, see De Robertis et al., 2000), understanding the mechanisms that limit organizer expansion is crucial for understanding embryonic patterning.

The organizer influences DV patterning through its secretion of BMP inhibitors such as Chordin (Chd) or Noggin (De Robertis et al., 2000). However, BMP also exerts its own effect on the organizer. The Xvent ventral homeobox genes were identified as transcriptional targets of BMP in *Xenopus*, and were shown to repress organizer gene expression on the ventral

side of the embryo (Gawantka et al., 1995; Onichtchouk et al., 1996; Onichtchouk et al., 1998; Melby et al., 1999; Lee et al., 2002). Indeed, Xvents repress the transcription of targets such as *chd* and *gooseoid* (*gsc*) (Onichtchouk et al., 1996; Melby et al., 1999; Trindade et al., 1999). Analysis of the *Xvent1b* and *Xvent2b* promoters revealed the presence of consensus Lef/Tcf binding sites (Friedle and Knöchel, 2002). In addition, the *Xvent1b* promoter is responsive to zygotic Wnt activity, suggesting that the expression of Xvent genes in general may be under the control of Wnt8 (Friedle and Knöchel, 2002). In support of this, Hoppler and Moon found that overexpression of dn-Xwnt8 leads to the reduction of both *Xvent1* and *Xvent2* expression in *Xenopus* (Hoppler and Moon, 1998). Thus, these studies suggest that the expression of transcriptional repressors required to restrict organizer gene expression may be under the concerted control of both the BMP and Wnt pathways.

Genetic analysis of zebrafish *vent* (also known as *vega2*, similar to *Xvent1*) and *vox* (also known as *vega1*, similar to *Xvent2*) showed that the proteins encoded by these genes function as redundant transcriptional repressors (Kawahara et al., 2000; Melby et al., 2000; Imai et al., 2001). Zebrafish embryos homozygous for a chromosomal deficiency of the closely linked *vent* and *vox* loci show an expansion of organizer gene expression and severe DV patterning defects (Imai et al., 2001). Further epistatic analysis suggested that the primary role of Vent and Vox is to modulate BMP inhibitors secreted by the organizer (Imai et al., 2001). *vent* and *vox* are known BMP transcriptional targets in zebrafish as well, but their dependency on BMP signaling starts at around 70-75% epiboly (Kawahara et al., 2000; Melby et al., 2000). As a result, zygotic BMP mutants do not have expanded organizers as *vent/vox* mutants do at shield stage (Mullins et al., 1996; Miller-

Bertoglio, 1997; Imai et al., 2001). To date, only two zebrafish zygotic mutants are known to display significantly expanded organizers: *vent/vox* mutants and *wnt8* mutants. These data suggest that the relationship between BMP, Wnt8 and Vent/Vox is an important one for organizer regulation, the nature of which has been unclear but has been suggested to be complex (Hoppler and Moon, 1998; Marom et al., 1999).

We have used a loss-of-function approach in zebrafish to study the relationship between Wnt8, zygotic BMP and Vent/Vox regulation and activity, in order to understand the mechanism by which Wnt8 antagonizes the organizer. Our results suggest that Wnt8 directly regulates the transcriptional levels of *vent* and *vox*, and that the maintenance of high levels of *vent* or *vox* is required for the repression of organizer genes on the ventral side of the embryo. Furthermore, we provide evidence that Vent and Vox are absolutely essential to mediate the organizer repression activity of Wnt8. We also show that organizer repression and the maintenance of ventrolateral mesoderm fates appear to be independent events. Finally, we show that the early regulation of both *vent* and *vox* is under Wnt8 and BMP control, but that Wnt8 is the primary regulator; that is, at the onset of gastrulation, the requirement for BMP is only revealed in the absence of Wnt8. Zygotic BMP becomes the primary regulator of *vent* (but not *vox*) transcription during mid to late gastrulation. Therefore, Wnt8 and BMP contribute to the repression of the organizer, which will, as a consequence, regulate the distribution of Wnt and BMP inhibitors.

Materials and methods

Fish maintenance and genetics

Animals were maintained as described (Westerfield, 2000). Embryos were staged according to Kimmel et al. (Kimmel et al., 1995). Our wild-type strain is AB. Mutants used were *Df(LG14)wnt8^{w8}* (Lekven et al., 2001), *Df^{ST17}* (Imai et al., 2001) and *swr^{TC300}* (Mullins et al., 1996). Results from *wnt8* or *vent/vox* deficiency mutants were confirmed with morpholinos (MOs).

In situ hybridization

In situ hybridizations were performed as described (Oxtoby and Jowett, 1993). Probes used were *gsc* (Stachel et al., 1993), *chd* (Miller-Bertoglio et al., 1997), *wnt8* ORF1 and *wnt8* ORF1+ORF2 (Lekven et al., 2001), *eve1* (Joly et al., 1993), *vent/vox* (Melby et al., 2000), *bmp2b* (Kishimoto et al., 1997), *opl* (Grinblat et al., 1998), *pax2a* (Krauss et al., 1991) and *tbx6* (Hug et al., 1997).

Genotyping of embryos

wnt8 mutants were genotyped as described (Lekven et al., 2001). *vent/vox* mutants were genotyped using *vox* R1 (5'-GATATTGCAC-ACCAGCGTGA-3') and *vox* L1 (5'-GTTCCAGAACCGAAGGATGA-3') primers. *swr* mutants were genotyped as described (Wagner and Mullins, 2002). Embryos were classified according to their phenotype, photographed and genotyped. For *wnt8;swr* double mutants, at least 85 embryos from an intercross were examined in the same fashion.

Embryo microinjection, morpholinos, constructs

MOs (Genetools, LLC), RNA or DNA were injected into one- to four-cell stage embryos. Approximately 3 nL was injected per embryo. Capped mRNAs were synthesized using mMACHINE mMESSAGE (Ambion) and diluted in water. MOs were diluted in Danieau's buffer as recommended (Genetools). *wnt8* MOs (targeting ORF1 and ORF2), and *vent* and *vox* MOs, have been described (Lekven et al., 2001; Imai et al., 2001). GR-LEFAN- β CTA RNA was injected at 300 ng/ μ L into

one-cell stage embryos. Embryos were dechorionated manually in fish water (Westerfield, 2000) prior to treatment. Dexamethasone (DEX; Sigma) treatments were performed for one hour at 1, 2, 3, 4 or 5 hours post-fertilization (HPF). DEX (100 mM stock solution in 100% ethanol) was used at a final concentration of 10 mM in 0.3 \times Danieau's solution. Treated embryos were fixed at 6 HPF. For the Cycloheximide (CHX; Calbiochem) treatment, embryos were first injected with GR-LEFAN- β CTA RNA then treated with CHX (10 μ g/mL), with or without DEX. For *vent* induction analysis, $n(\text{CHX})=37$ and 55, $n(\text{DEX})=44$, 37 and 11, and $n(\text{CHX+DEX})=28$, 34 and 28, where n =total number of embryos analyzed in each experiment. For *vox* induction, $n(\text{CHX})=16$, 17 and 12, $n(\text{DEX})=5$, 12 and 20, and $n(\text{CHX+DEX})=9$, 14 and 19. As a control for CHX treatments, uninjected embryos were treated with CHX from 1.5 HPF to sphere stage, then fixed and stained for *gsc* (Leung et al., 2003). No treated embryos expressed *gsc* ($n=34$). The χ^2 test was used to determine statistical significance.

Results

Zebrafish *wnt8* and *vent/vox* mutants have expanded organizers, *swr* mutants do not

Although BMP and Wnt8 both are described as 'ventralizing agents' (i.e. overexpression leads to a shift in mesodermal fates), they play non-equivalent roles in DV patterning. To illustrate this, we compared the expression of DV markers in *wnt8* (*Df^{w8}*) (Lekven et al., 2001), *vent/vox* (*Df^{st7}*) (Imai et al., 2001) and *bmp2b* (*swr^{tc300}*) (Mullins et al., 1996) mutants.

In zebrafish, *wnt8* contains two open reading frames (ORF1 and ORF2) (Lekven et al., 2001). The two Wnt8 proteins were shown to function redundantly in anteroposterior (AP) and DV patterning, as the *Df^{w8}* phenotype is phenocopied only by co-injection of both ORF1 and ORF2 MOs (Lekven et al., 2001). Similarly, the *Df^{st7}* phenotype is phenocopied by the co-injection of *vent* and *vox* MOs (Imai et al., 2001).

Expression analysis of the dorsal markers *chd*, *gsc*, *floating head* (*flh*) and *dharma* (*bozozok*) at shield stage shows that they are expanded ventrally in *wnt8* mutants (Fig. 1B,F) (Lekven et al., 2001) (and data not shown) as well as in *vent/vox* mutants (Fig. 1C, inset, and Fig. 1G) (Imai et al., 2001). *swr* mutants, however, do not exhibit a similar expansion at shield stage (Fig. 1D,H) (Mullins et al., 1996; Miller-Bertoglio et al., 1997). Importantly, the expansion of dorsal markers is stronger in *vent/vox* mutants than in *wnt8* mutants. For instance, *gsc* encircles the margin of *vent/vox* mutants (Fig. 1C, inset) but extends over a $\sim 90^\circ$ arc in *wnt8* embryos at the same stage (Fig. 1B). This comparative analysis shows that Wnt8 and Vent/Vox, but not BMP, are normally required ventrally during gastrulation to restrict the size of the organizer, which is in agreement with previous reports (Mullins et al., 1996; Miller-Bertoglio et al., 1997; Imai et al., 2001; Lekven et al., 2001).

The expanded organizer phenotype is first observed in *wnt8* embryos at 40% epiboly (discussed below), a developmental timepoint when convergence movements have not yet started (Kimmel et al., 1995). Thus, the expansion of dorsal markers in these backgrounds must reflect a change in fate rather than an alteration of cell movements.

Wnt8 is also required to promote ventral fates. *eve1*, a ventral mesodermal marker, is reduced in *wnt8* mutants (Fig. 1B). It is similarly reduced in *swr* mutants (Fig. 1D) (Mullins et al., 1996). By contrast, *eve1* is less reduced in *vent/vox* mutants (Fig. 1C) than in *wnt8* and *swr* mutants (Fig. 1B,D),

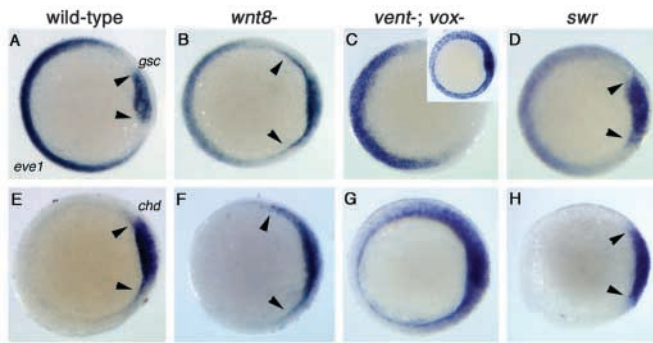


Fig. 1. The *wnt8*⁻ phenotype is similar to the *vent*⁻; *vox*⁻ and *swr* phenotypes. (A,B,D) Double in situ hybridization for *eve1* and *gsc*. (C) *eve1* expression, inset shows *gsc*. Note strongly reduced *eve1* in *wnt8* and *swr* mutants but only slightly reduced *eve1* in *vent*; *vox* mutants. Arrowheads indicate the width of *gsc* expression (note circumferential *gsc* in C, inset). (E-H) In situ hybridization for *chd* (domain width indicated by arrowheads). Note expansion in both *wnt8* and *vent*; *vox* mutants, but not *swr* mutants. All embryos are at shield stage. Animal view, dorsal right.

despite the fact that the dorsal markers *gsc* (Fig. 1C, inset) or *chd* (Fig. 1G) encircle the margin of the same embryos. Hence, Wnt8 and BMP are required in the ventral mesoderm for the maintenance of *eve1*, a ventral-specific gene, and this function is separable from repression of the organizer.

Wnt8 regulates *vent* and *vox* mRNA levels

Because Wnt8 and Vent/Vox share the function of repressing dorsal genes, we analyzed their epistatic relationship. We first examined *vent* and *vox* mRNA levels in wild-type versus *wnt8*⁻ backgrounds (Fig. 2). In zebrafish, *vent* is expressed at the mesodermal margin during gastrulation, whereas *vox* displays both ventral mesoderm and ectoderm expression (Melby et al., 2000).

Starting at 30% epiboly (late blastula), the accumulation of *vent* at the margin is visibly weaker in *wnt8* mutants or morphants than in wild type (Fig. 2A-C). We did not detect any

differences in *vent* expression at earlier stages (data not shown). *vox* expression is not visibly different in *wnt8* mutants at 30% epiboly (data not shown), but is reduced in the margin of *wnt8* mutants/morphants at 40% epiboly (Fig. 2G-I).

To determine the correspondence between *vent* and *vox* reduction and the onset of an observable phenotype in *wnt8* mutants, we examined *chd* expression at these early stages. At 30% epiboly, no visible difference in the *chd* expression domain was observed in *wnt8* mutants (data not shown), but we did detect an expansion of *chd* expression at 40% epiboly, the timepoint at which both *vent* and *vox* are reduced in *wnt8*⁻ embryos (Fig. 2M-O). Hence, our results suggest that a reduction in both *vent* and *vox* levels may be required to observe the expanded organizer phenotype at 40% epiboly, which is consistent with Vent and Vox functioning redundantly (Imai et al., 2001).

During the rest of gastrulation, *vent* and *vox* mRNA levels stay reduced in *wnt8* mutants/morphants compared with in wild type (Fig. 2D-F, J-L; data not shown). By comparison, *vent* and *vox* levels are unchanged in *swr* mutants at shield stage (Kawahara et al., 2000; Melby et al., 2000), which explains the lack of an organizer phenotype (Mullins et al., 1996; Miller-Bertoglio et al., 1997). Indeed, *Bmp2b* is only required at mid to late gastrulation for the maintenance of *vent* and ectodermal *vox* expression (Melby et al., 2000). Therefore, Wnt8 regulation of *vent* and *vox* starts at the blastula/gastrula transition (30/40% epiboly), whereas *Bmp2b* regulation of these genes occurs later (70% epiboly).

To test the reciprocal possibility of *wnt8* being regulated by Vent and Vox, we looked at the expression of *wnt8* in *vent*; *vox* mutants (Fig. 3). As zebrafish *wnt8* produces transcripts for both protein coding regions, we used probes to detect either the ORF1/ORF2 bicistronic transcript (ORF1), or both the bicistronic transcript and the ORF2 transcript (ORF1+ORF2) (Lekven et al., 2001). No differences from wild-type expression were observed in 30% or 40% epiboly *vent*; *vox* mutants (Fig. 3A,B,G,H). Because *vent*; *vox* mutants are affected prior to 30% epiboly (Imai et al., 2001), this suggests that a change in *wnt8* expression is not responsible for the *vent*; *vox* mutant phenotype. The dorsal domain lacking ORF1

Fig. 2. *vent* and *vox* mRNA levels are reduced in *wnt8* mutants. In situ hybridization for *vent* (A-F), *vox* (G-L) or *chd* (M-R). Embryo genotypes are indicated above each column; stages are also indicated. At 30% epiboly, *vent* expression is reduced in *wnt8* mutants/morphants (arrows in B,C). *vox* is reduced at 40% epiboly (arrows in H,I), corresponding to increased *chd* expression (arrowheads in N,O). Both *vent* (E,F) and *vox* (K,L) expression are reduced in shield stage *wnt8* mutants/morphants. Animal view, dorsal right.

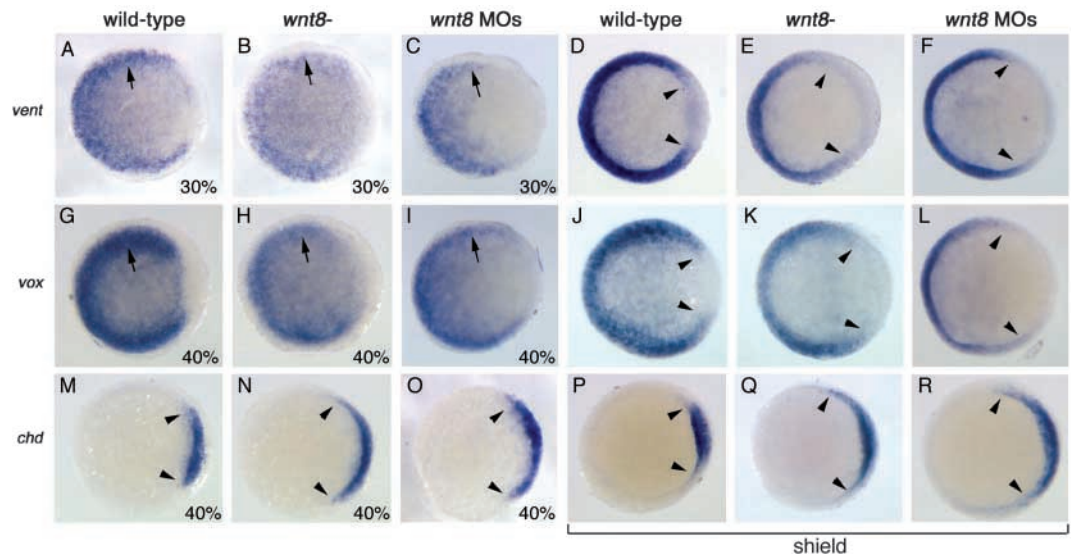
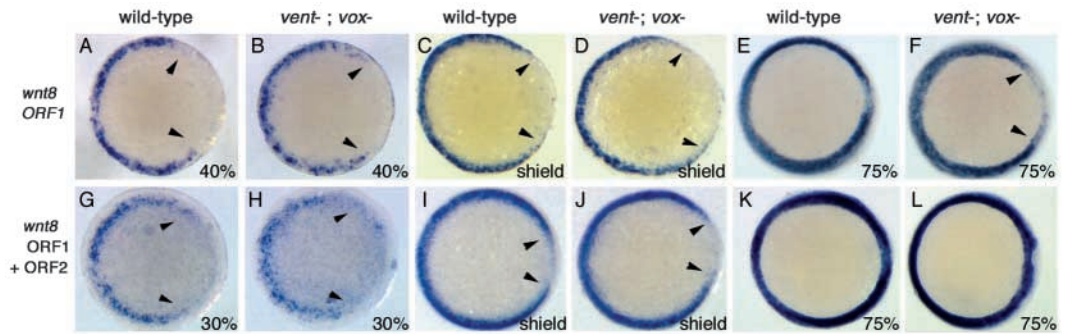


Fig. 3. *Wnt8* ORF1 and ORF2 expression in *vent;vox* mutants. In situ hybridization for *wnt8* ORF1 (A-F) and *wnt8* ORF1+ORF2 (G-L). Genotypes are indicated above each column; stages are also indicated. Arrowheads indicate the dorsal limit of *wnt8* expression. Note the slight decrease in ORF1 dorsally in shield stage *vent;vox* mutants (C,D), and the broadened dorsal clearing of *wnt8* ORF1 expression at 75% epiboly (F). *wnt8* ORF2 expression is not affected. (A-D,G-J) Animal view, dorsal right. (E,F,K,L) Vegetal view, dorsal right.



expression is slightly expanded in *vent;vox* mutants at shield stage (Fig. 3C,D; confirmed with MOs) and is more pronounced at 75% epiboly (Fig. 3F). Although there is an observable difference dorsally, ORF1 levels ventrally seem to be unaffected in *vent;vox* mutants (Fig. 3C-F), suggesting that the reduction in dorsal *wnt8* ORF1 expression is an indirect consequence of an enlarged organizer. Analysis of ORF2 expression at later stages revealed that it is not affected by the loss of Vent and Vox (Fig. 3I-L). This is not unexpected as *wnt8* ORF2 accumulates dorsally during gastrulation (Fig. 3K) and is therefore insensitive to molecules present in the organizer. Thus, only *wnt8* ORF1 expression depends on Vent and Vox, but this dependency is restricted dorsally and may be indirect. By comparison, *wnt8* ORF2 expression does not depend on Vent and Vox.

Wnt8 functions through β -catenin to regulate *vent* and *vox* transcription

The above data show that Wnt8/ β -catenin is necessary to maintain normal *vent* and *vox* expression. To test whether Wnt8 is sufficient to induce *vent* and *vox*, we injected Wnt8 ORF1 or ORF2 expression plasmids into wild-type embryos and assayed *vent* and *vox* expression by in situ hybridization at shield stage. In both cases, ectopic domains were observed in the animal ectoderm region and/or dorsal mesoderm, where *vent* and *vox* are normally absent (Table 1, and data not shown). To confirm that canonical Wnt signaling was involved in *vent* and *vox* regulation, we modulated β -catenin activity using a hormone inducible β -cat/Lef fusion protein (GR-LEF Δ N- β CTA) (Domingos et al., 2001). The GR-LEF Δ N- β CTA protein contains the human glucocorticoid receptor domain fused to the DNA-binding domain of murine LEF and the transactivation domain of murine β -catenin. Addition of the hormone dexamethasone (DEX) leads to the nuclear translocation of the fusion protein and to β -catenin/Lef-induced transcription, thus allowing controlled induction of Wnt signaling (Domingos et al., 2001). Addition of DEX for a one-hour period at 1, 2, 3, 4 or 5 HPF led to ectopic *vent* and *vox* expression in a proportion of injected embryos (~50-70% of embryos; Fig. 4A, panels b,d; data not shown). Consistent with the role of β -catenin in organizer induction, ectopic *gsc* was observed in a proportion of embryos treated at 1, 2 or 3 HPF, but not at later timepoints (data not shown).

Although our results suggest that Wnt8/ β -catenin regulates *vent* and *vox* transcription, it is unclear whether this is direct (through β -catenin/Lef-induced transcription) or indirect

(through the synthesis of an intermediate transcriptional regulator). Interestingly, the genomic region upstream of zebrafish *vox* contains consensus Lef/Tcf binding sites consistent with Wnt regulation of *vox* transcription (our own observations, and D. Kimelman, personal communication). To address this, we used cycloheximide (CHX) to test whether protein synthesis is required for the induction of ectopic *vent* or *vox* by GR-LEF Δ N- β CTA. Treatment of GR-LEF Δ N- β CTA-injected embryos with DEX at 5 HPF results in ectopic *vent* or *vox* RNA expression in 49% and 62.1% of embryos, respectively (Fig. 4B). Addition of CHX simultaneously with DEX did not result in a statistically different number of embryos with ectopic *vent* and *vox* domains (72.2% and 59.5%; Fig. 4B), indicating that GR-LEF Δ N- β CTA activation of *vent* and *vox* does not require de novo protein synthesis. Thus, our results suggest that *vent* and *vox* are direct transcriptional targets of Wnt8/ β -catenin signaling.

Wnt8 repression of the organizer requires Vent/Vox

As *vent* and *vox* transcription is regulated by Wnt8, we hypothesized that Vent and Vox function downstream of Wnt8 to repress dorsal genes, and that the *wnt8*⁻ organizer phenotype is due to reduced *vent* and *vox* levels. If this is correct, injection of *vent* or *vox* RNA or DNA into *wnt8* mutants would suppress the expanded organizer phenotype. We first established amounts of injected Vox or Vent that are sufficient to reduce the expression of dorsal markers (*gsc*, *chd*, *flh*) in wild-type embryos (Fig. 5A, panels a,c; data not shown). When injected into *wnt8* mutants, Vox was able to reduce the expression of dorsal genes (Fig. 5A, compare panels b and d; Table 2). Similar results were obtained with either DNA or RNA injection for both *vent* and *vox* (Table 2, and data not shown). Thus, Vent and Vox expression can bypass *wnt8* loss-of-function in repressing organizer genes, thus supporting the placement of *vent* and *vox* genetically downstream of *wnt8*.

Table 1. Injection of either *wnt8* ORF induces ectopic *vent* and *vox* expression

Injection	Assay	% Injected embryos showing ectopic expression	P value
<i>wnt8</i> ORF1 DNA (40 ng/ μ L)	<i>vent</i>	75.0 (n=56)	<0.001
	<i>vox</i>	73.0 (n=52)	<0.001
<i>wnt8</i> ORF2 DNA (40 ng/ μ L)	<i>vent</i>	91.8 (n=49)	<0.001
	<i>vox</i>	89.1 (n=37)	<0.001

Fig. 4. *vent* and *vox* are direct transcriptional targets of Wnt8/ β -catenin signaling. (A) *vent* and *vox* expression in control (a,c) or treated (b,d) embryos. Arrows in panels b and d indicate ectopic expression upon induction of GR-LEF Δ - β CTA with DEX. (B) Percentage of embryos displaying ectopic *vent* or *vox* domains (y-axis) upon treatment with CHX alone, DEX alone, or CHX+DEX (x-axis). The control bar represents embryos injected with GR-LEF Δ - β CTA and treated with ethanol ($n=109$ for *vent*, $n=177$ for *vox*). Error bars represent s.e.m. When performing the χ^2 test on DEX versus DEX+CHX means, $P>0.05$ for both *vent* and *vox*, meaning that the difference between the means is not statistically significant.

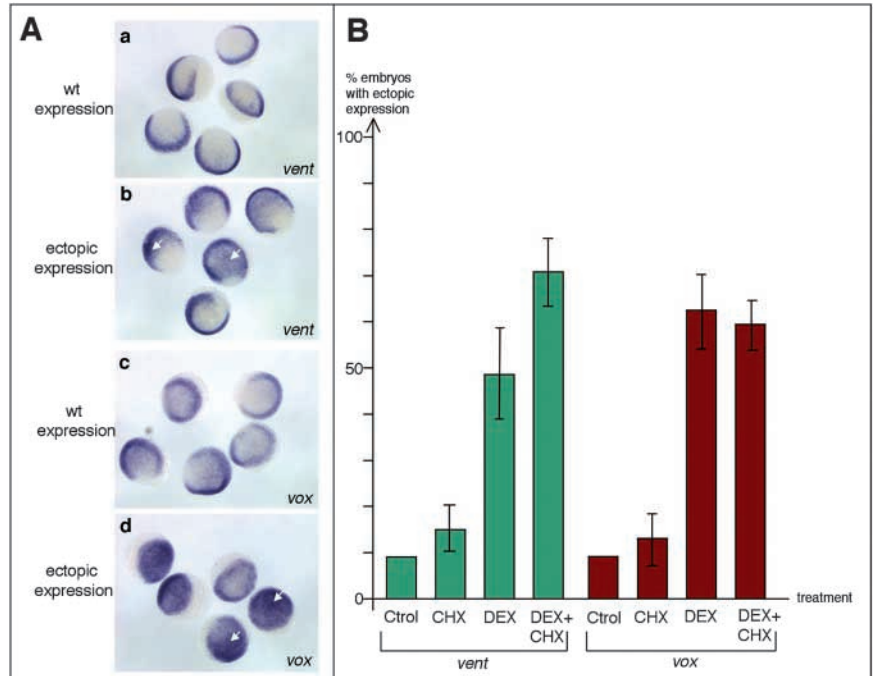
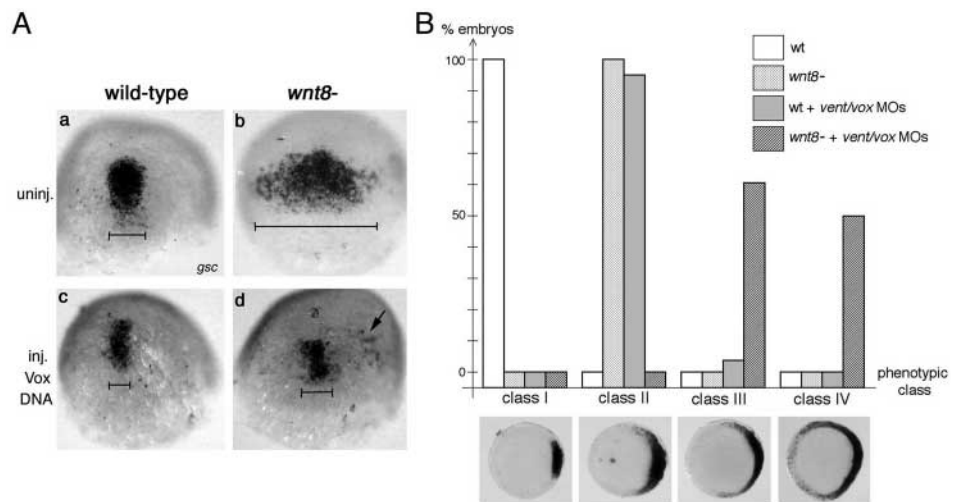


Fig. 5. The *wnt8*⁻ expanded organizer phenotype is due to reduced *vent* and *vox* expression. (A) Rescue of *wnt8* mutants by *Vox*. *gsc* expression (bracket) in wild-type (a,c) or *wnt8*⁻ (b,d) embryos, uninjected (a,b) or injected (c,d) with a *vox* expression plasmid. Some isolated lateral cells still express *gsc* in injected *wnt8*⁻ embryos because of the mosaic expression of *Vox* (panel d, arrow). Embryos shown are at 70% epiboly, dorsal view. (B) Reduction of *Vent/Vox* enhances the *wnt8*⁻ organizer phenotype. Graph shows the percentage of embryos belonging to a specific phenotypic class. Class I, wild-type *chd* expression; class II to IV, increasingly expanded *chd* expression. 100% of wild-type and *wnt8*⁻ embryos belong to class I and class II, respectively. Upon injection of *vent+vox* MOs, most wild-type embryos belong to class II (96.5%, $n=85$), whereas *wnt8*⁻ embryos belong to both classes III and IV (60.7% and 39.3%, respectively, $n=28$). Embryos shown at the bottom of the graph are at shield stage, animal view, dorsal right.



These results suggest that the difference in severity of the *wnt8*⁻ and *vent*⁻;*vox*⁻ organizer phenotypes (see Fig. 1) could be explained by residual *Vent* and *Vox* activity in *wnt8* mutants. In agreement with this, further reduction of *Vent* and *Vox* in *wnt8* mutants by injection of sub-maximal concentrations of *vent* and *vox* MOs enhances the severity of the *wnt8*⁻ phenotype (Fig. 5B).

While *Vent* and *Vox* can bypass Wnt8 to repress organizer genes, we wished to assess whether Wnt8 requires *Vent* and *Vox* to repress the organizer. If *Vent* and *Vox* are essential for this Wnt8 function, then Wnt8/ β -catenin activity should be ineffective in their absence. In support of this, *vent*;*vox* mutants express nearly normal levels of *wnt8* mRNA (see Fig. 3), hence

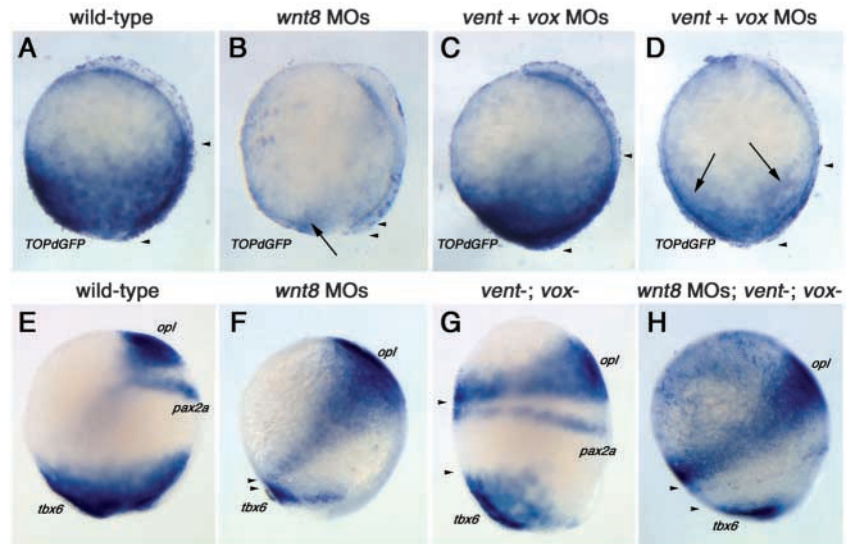
the expansion of the organizer in *vent*;*vox* mutants occurs in the presence of *wnt8* transcripts.

To confirm that the *wnt8* transcripts in *vent*;*vox* mutants produce functional proteins, we used two assays of Wnt8 function. First, we examined the expression of the Wnt/ β -catenin activity reporter TOPdGFP (Dorsky et al., 2002; Lewis et al., 2004). We analyzed the expression of TOPdGFP mRNA at 100% epiboly in embryos homozygous for the transgene after injection of *wnt8* or *vent+vox* MOs (Fig. 6A-D). As expected, and confirming previous results (Phillips et al., 2004), *wnt8* MOs severely reduce TOPdGFP expression in 90% of injected embryos to almost undetectable levels ($n=20$; Fig. 6B). In *vent/vox* morphants, three phenotypic classes were

Fig. 6. Wnt8 requires Vent and Vox to repress dorsal genes. (A-D) GFP in situ hybridization to embryos homozygous for the TOPdGFP transgene. (E-H) *opl*, *pax2a* and *tbx6* in situ hybridization.

Genotype/treatment is indicated above each panel. (A) TOPdGFP is expressed in the mesoderm. In *wnt8* morphants (B), TOPdGFP is barely detectable (arrow). *vent+vox* MO-injected embryos display mostly wild-type TOPdGFP expression (C), but some display somewhat reduced expression (D, arrows). Arrowheads in A-D indicate the AP extent of the TOPdGFP positive domain. (E) In wild type, *opl* and *pax2a* expression domains in relation to *tbx6* indicate normal neural posteriorization. In *wnt8* morphants, *opl* is expanded posteriorly, *pax2a* is delayed and *tbx6* is reduced (F).

vent;vox mutants (G) do not display a strong AP defect, and ventral *tbx6* staining is as strong as in wild-type embryos. Reducing Wnt8 in *vent;vox* mutants (H) results in decreased *tbx6* and *pax2a* expression. The distance between the arrowheads in F, G and H show the degree of posteriorization. Embryos shown are at ~100% epiboly, lateral view, dorsal right.



observed: the first class displayed wild-type TOPdGFP expression (50%, $n=22$; Fig. 6C); the second class showed moderate reduction in TOPdGFP (14%, not shown); and the third class displayed a stonger reduction in staining (36%; Fig. 6D), but this class had significantly more TOPdGFP expression than *wnt8* morphants (compare Fig. 6D to Fig. 6B). As a control for the strength of the *vent+vox* MO injections, a sample of the injected embryos was examined at 24 HPF and all showed a strong *vent/vox* loss-of-function phenotype ($n=23$) (Imai et al., 2001). Thus, TOPdGFP is a reporter of Wnt8 activity and is still expressed in *vent+vox* morphants. Reduced levels of TOPdGFP expression in some *vent+vox* morphants could reflect the fact that expression of the Wnt antagonists Dickkopf 1 and Frzb is significantly expanded (Imai et al., 2001) (and our own observations).

To confirm that expressed Wnt8 actively patterns *vent;vox* mutants, we analyzed AP neural patterning, a function known to require Wnt8 (Lekven et al., 2001; Erter et al., 2001). To assess the AP phenotype of *vent;vox* mutants, a combination of three probes was used: *opl* (anterior neuroectoderm), *pax2a* (midbrain-hindbrain border) and *tbx6* (posterior non-axial mesoderm). In *wnt8* mutants or morphants, AP patterning is severely disrupted at 90%-100% epiboly: the *opl* domain is expanded along the AP axis, *pax2a* expression is delayed and

tbx6 expression is strongly reduced (Fig. 6F). By comparison, *vent;vox* mutants have only mildly affected AP patterning illustrated by a slight posterior shift of the *opl* and *pax2a* domain away from the animal pole, but the distance between *opl* or *pax2a* and *tbx6* is significantly greater than in *wnt8* morphants (Fig. 6G, compare with Fig. 6F). As expected, the expanded organizer of *vent;vox* mutants results in an enlarged dorsal clearing of *tbx6* expression, whereas the levels of *tbx6* ventrally are relatively unaffected (Fig. 6G, compare with Fig. 6E). As *tbx6* expression depends on Wnt8, our results do not support an absence of Wnt8/ β -catenin activity in *vent;vox* mutants. Furthermore, reducing Wnt8 translation in *vent;vox* mutants results in an additive phenotype. *opl* extends ventrally, as in *vent;vox* mutants, whereas *pax2a* and *tbx6* expression is severely reduced, as in *wnt8* mutants (Fig. 6H). Taken together, these results show that Wnt8 expression and patterning activity does not depend on Vent and Vox, with the significant exception that Wnt8 is unable to repress organizer genes when Vent and Vox are absent.

To further show that Wnt8 requires Vent and Vox in organizer repression, we tested whether exogenous Wnt8 can repress organizer genes in *vent;vox* mutants. We injected a *wnt8* ORF1 expression plasmid (20 ng/ μ L) into one-cell stage *vent;vox* mutants and assayed *gsc* expression at shield stage. No injected *vent;vox* mutant embryos ($n=25$; genotyped by PCR) displayed reduced *gsc* expression, although this treatment did result in decreased *gsc* expression in wild-type siblings ($n=54$). As a control, we checked that the injected *wnt8* DNA was sufficient to induce ectopic *vent* and *vox* expression in wild-type embryos (64% ectopic expression for *vent*, $n=25$; 42.8% ectopic expression for *vox*, $n=35$). Thus, repression of the organizer by exogenous Wnt8 requires Vent or Vox.

Our results show that in the absence of Vent and Vox, *wnt8* is expressed and is active, as assayed by TOPdGFP reporter expression, *tbx6* expression and embryonic AP patterning. Furthermore, ectopic Wnt8 cannot repress *gsc* in *vent;vox* mutants. These data strongly support a linear model in which

Table 2. Increased Vent/Vox expression in *wnt8* mutants leads to the repression of dorsal genes

Assay	Injection (10 ng/ μ L)	% Rescued <i>wnt8</i> mutants*	P value
<i>gsc</i>	<i>vox</i> DNA	53.3 ($n=15$)	<0.001
	<i>vent</i> RNA	78.9 ($n=19$)	<0.001
<i>chd</i>	<i>vox</i> RNA	68.7 ($n=16$)	<0.001
<i>flh</i>	<i>vox</i> RNA	95.4 ($n=22$)	<0.001

*Rescue is defined as a reduction in the dorsal markers assayed compared with in uninjected *wnt8* mutants.

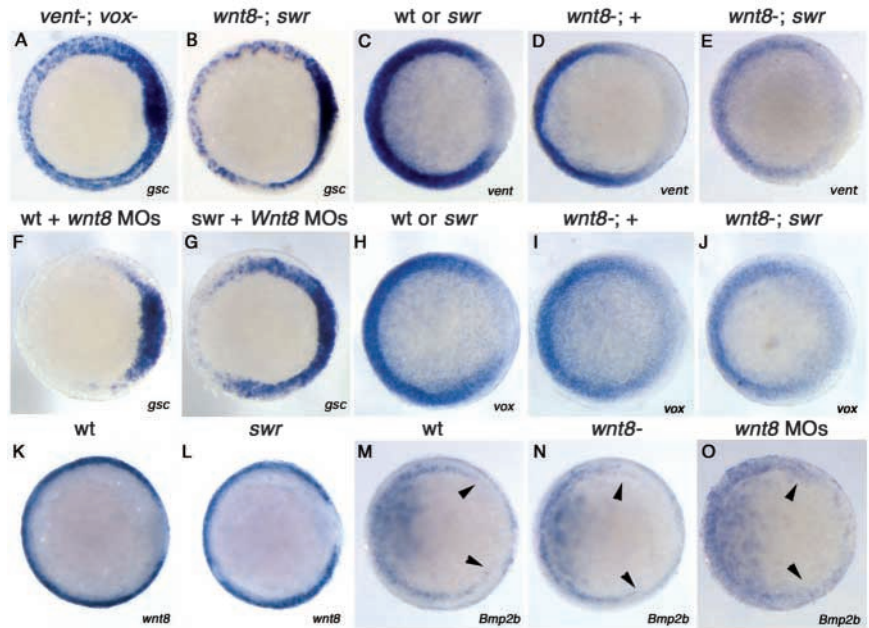


Fig. 7. Wnt8 and zygotic BMP both regulate *vent* and *vox*, but do so differently. In situ hybridization for *gsc* (A,B,F,G), *vent* (C-E), *vox* (H-J), *wnt8* (K,L) and *bmp2b* (M-O). Genotypes/treatments are indicated above each panel. Note circumferential *gsc* in *vent;vox* (A) and *wnt8;swr* (B,G) double mutants/morphants, and the strong reduction of *vent* (E) and *vox* (J) in *wnt8;swr*⁻. *wnt8* is still expressed in *swr* mutants (L), and *bmp2b* is still expressed in *wnt8* mutants/morphants (N,O). Arrowheads in M-O indicate the dorsal limits of mesodermal *bmp2b*, which is shifted slightly ventrally in *wnt8* mutants/morphants (N,O). Embryos shown are at shield stage, animal view, dorsal right.

Wnt8 acts directly upstream of Vent and Vox to repress the organizer.

Both Wnt8 and Bmp2b are required at different timepoints for the maintenance of *vent* and *vox*

Two pathways are required for the maintenance of *vent* and *vox* expression in zebrafish: the zygotic BMP pathway (Melby et al., 2000; Imai et al., 2001) and the Wnt pathway (this work). To understand the combined regulation of *vent* and *vox* during gastrulation by the Wnt8 and BMP pathways, we analyzed the phenotype of *wnt8;swr* double mutants (Fig. 7). Using *swr* (*bmp2b*) mutants is sufficient to assess the influence of zygotic BMP signaling, as it was previously shown that loss of Bmp2b produces a zygotic *bmp*⁻ null phenotype (Schmid et al., 2000). The requirement for both BMP and Wnt8 inputs towards *vent* and *vox* expression would be revealed if *wnt8;swr* double mutants exhibit a phenotype similar to the *vent;vox*⁻ phenotype. We found that *gsc* and *chd* are expressed in a broader domain around the mesodermal margin in shield stage *wnt8;swr* double mutants compared with either single mutant (Fig. 7B, compare with Fig. 1; data not shown), and thus they phenocopy *vent;vox* mutants (Fig. 7A). The same results were obtained when using the *wnt8* deficiency or *wnt8* MO knockdown (Fig. 7G), confirming the specificity of the interaction.

As *wnt8;swr* double mutants display the same expanded organizer phenotype as *vent;vox* mutants at shield stage, we expected *vent* and *vox* mRNAs to be either absent or strongly reduced. We found both *vent* and mesodermal *vox* to be strongly reduced but not completely absent in shield stage *wnt8;swr* double mutants (Fig. 7E,J). Both *vent* and *vox* transcripts are not detectable in the mesoderm of later stage *wnt8;swr* double mutants (data not shown).

The fact that double mutants appear to be worse than *wnt8* or *swr* single mutants suggests that Wnt8 and BMP function in parallel to regulate *vent* and *vox*. Consistent with this, *bmp2b* expression in *wnt8* mutants/morphants is close to wild type (Fig. 7M-O), and *wnt8* expression in *swr* mutants is normal at

shield stage (Fig. 7K,L). Hence, both Wnt8 and Bmp2b are early regulators of *vent* and *vox*, but Wnt8 has a more prominent role until mid-gastrula stages.

Discussion

To understand the DV phenotype of *wnt8* mutants, we have analyzed the interaction of Wnt8, BMP, Vent and Vox. We found that the levels of both repressors are lower in *wnt8*⁻ embryos at 40% epiboly when the expanded organizer phenotype initiates (Fig. 8). Consistent with a direct role for Wnt8 in *vent/vox* regulation, an inducible Lef/ β -catenin fusion protein induces ectopic *vent* and *vox* transcription in the absence of new protein synthesis. Vent and Vox can repress organizer genes in the absence of Wnt8, suggesting that a simple linear pathway connects Wnt8/ β -catenin with Vent/Vox-dependent organizer repression. In support of this, Wnt8 is unable to repress the organizer in the absence of Vent and Vox, although it is able to induce a Wnt reporter gene and to function in AP patterning. In addition, exogenous Wnt8 cannot repress *gsc* in *vent;vox* mutants. Finally, *vent* and *vox* regulation is under the control of both Wnt8 and zygotic BMP (Fig. 8), although Wnt8 is the primary regulator during early- to mid-gastrula stages.

vent and *vox* are transcriptional targets of Wnt8/ β -catenin signaling

Although it is not known what induces *vent* and *vox*, our data show that Wnt8 regulates their early transcriptional maintenance. What is unclear is which Lef or Tcf proteins are involved in Wnt8-mediated transcriptional regulation. Studies in *Xenopus* suggest that Lef1 and not Tcf3 may mediate Xwnt8 function (Roel et al., 2002), but this has not yet been addressed in zebrafish.

Interestingly, it has recently been observed that overexpression of a conditional dominant repressor form of Tcf (hs- Δ Tcf) leads to a more severe phenotype than the loss of Wnt8 (Lewis et al., 2004). Lewis et al. found that *gsc* expression encircles the margin of transgenic hs- Δ Tcf embryos

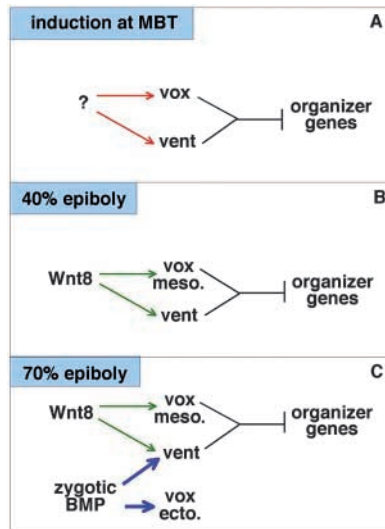


Fig. 8. Regulation of *vent* and *vox* by Wnt8 and zygotic BMP. (A) *vent* and *vox* are induced around MBT by an unknown factor. (B) At 40% epiboly, Wnt8 is required to maintain high levels of *vent* and mesodermal *vox* expression. (C) At 70% epiboly, in addition to Wnt8, zygotic BMP is required to maintain *vent* expression. BMP is also required for ectodermal *vox* expression. Thicker arrows represent stronger regulatory connections, as *vent* and ectodermal *vox* expression is absent in zygotic BMP mutants at this stage, whereas *vent* and mesodermal *vox* expression are only reduced in *wnt8* mutants.

heat-shocked at 4 HPF, a phenotype similar to *vent;vox* or *wnt8;swr* double mutants. Why would overexpression of a dominant-negative Tcf produce a more severe phenotype than loss of Wnt8 signaling? This could be explained if Δ Tcf not only abolishes Wnt8 function but also prevents other factors from positively regulating *vent* and *vox*. One such factor could be the Smads that mediate Bmp2b function, as we have shown that zygotic BMP signaling is essential for maintaining *vent* and *vox* expression in the absence of Wnt8. In other words, Δ Tcf may prevent Smad-dependent regulation of *vent* and *vox*.

Regulation of *vent* and *vox* by Wnt8: comparison between zebrafish and *Xenopus*

The transcriptional regulation of *Xvent* genes has been studied quite extensively in *Xenopus*, where most were found to be direct targets of Bmp4 signaling (Rastegar et al., 1999; Henningfeld et al., 2000; Henningfeld et al., 2002; Lee et al., 2002). However, the analysis of their regulation by Xwnt8 is less complete. It was found that zygotic Wnt signaling is necessary and sufficient for *Xvent1* and *Xvent2* expression (Hoppler and Moon, 1998; Marom et al., 1999), in agreement with our findings for zebrafish Wnt8. Analysis of *Xenopus* embryos overexpressing dominant-negative *Xvent1* and *Xvent2* revealed that *Xwnt8* expression is not affected by the loss of *Xvent* activity (Onichtchouk et al., 1998). Again, our data agree as *wnt8* is expressed in *vent;vox* mutants. The inability of Xwnt8 to rescue the dominant-negative *Xvent* phenotype was interpreted to mean that Xwnt8 functions in a different pathway than Bmp4/*Xvent* (Onichtchouk et al., 1998). However, we propose that, as in zebrafish, Xwnt8 functions upstream of *Xvent* genes, and that apparent

differences between our model and *Xenopus* models may be due to the different experimental approaches. For example, concomitant reduction of Xwnt8, and *Xvent1* and *Xvent2*, activities using dominant-negative proteins results in a more severe phenotype than reducing *Xvent1* and *Xvent2* alone (Onichtchouk et al., 1998). This is also what we observed when injecting *vent* and *vox* MOs in a *wnt8*⁻ background. Thus, our results agree with data obtained in *Xenopus*, although our interpretation of the Wnt8/Vent/Vox relationship is somewhat different.

Wnt8 and zygotic BMP are required during gastrulation to maintain *vent* and *vox* expression at different timepoints

Our results show that both Wnt8 and Bmp2b (hence zygotic BMP) are required to maintain *vent* and *vox* levels during gastrulation, but that Wnt8 regulation of those genes occurs earlier at the blastula/gastrula transition (Fig. 8). The lack of an expanded organizer in *swr* mutants can be explained by the late regulation of *vent* and *vox* by zygotic BMP after the organizer has been formed. In addition, mesodermal *vox* levels are unchanged in *swr* mutants (only ectodermal *vox* levels are reduced at 70%) (Melby et al., 2000). Hence, mesodermal Vox can repress dorsal genes in *swr* mutants. Consistent with this, injection of *vox* MO in *swr* mutants results in expanded *gsc* expression at 70% epiboly (M.-C.R. and A.C.L., unpublished).

There are two known BMP signaling pathways in *Xenopus* and zebrafish (Dale and Jones, 1999; Wilm and Solnica-Krezel, 2003). In zebrafish, the maternal BMP pathway is thought to establish ventral identity in a manner analogous to the establishment of a dorsal axis by maternal β -catenin activity (Kramer et al., 2002; Sidi et al., 2003). Understanding the regulation of Wnt8 by maternal and zygotic BMP may explain apparently contradictory results from *Xenopus* and zebrafish. For instance, whereas it was found that regulation of zebrafish *vent* and *vox* by zygotic BMP occurs at mid to late gastrulation (Melby et al., 2000), *Xenopus Xvent2* regulation by BMP signaling occurs during early gastrulation (stage 10.5) (Ladher et al., 1996). *Xvent2* regulation was observed in embryos overexpressing a truncated Bmp2/4 receptor that does not distinguish between Bmp2 or Bmp4 ligands (Suzuki et al., 1994). However, Bmp2 is both maternally provided and zygotically expressed (Dale and Jones, 1999). It has therefore been suggested that *Xvent2* expression may be under the influence of a maternal BMP signal (Ladher et al., 1996). Interestingly, the use of the same BMP-knockdown approach also results in decreased *Xwnt8* expression (Schmidt et al., 1995; Hoppler and Moon, 1998). In zebrafish, it has been reported that loss of maternal BMP (Radar) signaling does not interfere with the induction of *vent* and *vox* at MBT (Sidi et al., 2003), although embryos homozygous for maternal *smad5* display slightly expanded *gsc* and *chd* expression (Kramer et al., 2002). Thus, the elucidation of the relationship between Wnt8 and maternal or zygotic BMP in zebrafish using a loss-of-function approach may address whether the regulation of *vent* and *vox* is fundamentally different between zebrafish and *Xenopus*.

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